Selectivity of diacylhydrazine insecticides to the predatory bug *Orius laevigatus: in vivo* and modelling/docking experiments

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Abstract

BACKGROUND: Knowledge of pesticide selectivity to natural enemies is necessary for a successful implementation of biological and chemical control methods in integrated pest management (IPM) programmes. Diacylhydrazine (DAH)-based ecdysone agonists, also known as moulting-accelerating compounds (MACs), are considered to be a selective group of insecticides, and their compatibility with predatory Heteroptera, which are used as biological control agents, is known. However, their molecular mode of action has not been explored in beneficial insects such as *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae).

RESULTS: In this project, *in vivo* toxicity assays demonstrated that the DAH-based RH-5849, tebufenozide and methoxyfenozide have no toxic effect against *O. laevigatus*. The ligand-binding domain (LBD) of the ecdysone receptor (EcR) of *O. laevigatus* was sequenced, and a homology protein model was constructed that confirmed a cavity structure with 12 α-helices, harbouring the natural insect moulting hormone 20-hydroxyecdysone. However, docking studies showed that a steric clash occurred for the DAH-based insecticides owing to a restricted extent of the ligand-binding cavity of the EcR of *O. laevigatus*.

CONCLUSIONS: The insect toxicity assays demonstrated that MACs are selective for *O. laevigatus*. The modelling/docking experiments are indications that these pesticides do not bind with the LBD-EcR of *O. laevigatus* and support the supposition that they show no biological effects in the predatory bug. These data help in explaining the compatible use of MACs together with predatory bugs in IPM programmes.

Keywords: *Orius laevigatus*; selectivity; diacylhydrazine insecticides; ecdysone receptor; homology modelling; docking studies

1 INTRODUCTION

Biological control is considered a key strategy in sustainable agriculture. Flower bugs in the genus *Orius* are important natural enemies of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), and they are commonly released in North American and European sweet pepper greenhouses.[1,2] *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) is widely spread around the Mediterranean area and the north-west of Europe[3] and feeds on western flower thrips and various insect pests from several orders, including *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and some phytophagous mites.[4,5] Within the framework of an environmentally friendly integrated crop protection strategy, selective insecticides should be compatible with the natural enemies used. Therefore, knowledge of the activity of insecticides towards beneficial insects is needed.

Diacylhydrazine (DAH)-based insecticides such as RH-5849, tebufenozide and methoxyfenozide, also known as moulting-accelerating compounds (MACs), are more selective compared with the conventional groups owing to their interference with specific insect targets, such as the insect endocrinological pathways. Their activity is based on binding to the receptor site of the insect moulting hormone 20-hydroxyecdysone (20E), the ecdysone receptor (EcR), and thus inducing premature lethal moulting in larval stages and aborting reproduction in adults, especially in Lepidoptera and Coleoptera.[6–8] Moreover, deleterious effects have also been reported on mosquito larvae of *Aedes aegypti* (L.), *Culex quinquefasciatus* (Say) and *Anopheles gambiae* (Giles) (all Diptera: Culicidae).[9]

The EcR is a nuclear hormone receptor present in all arthropods.[10,11] The basic structure of typical nuclear receptors includes several modular domains: an N-terminal region (A/B do-

main), a DNA-binding domain (DBD, C domain), a hinge region (D domain), a ligand-binding domain (LBD, E domain) and in some cases also a C-terminal F domain.[12] The A/B, D and F domains are usually poorly conserved, and their structure is unknown. In contrast, the DBD and LBD are highly conserved, and their structure has been determined for several receptors.[13] The DBD contains two typical cysteine-rich zinc finger motifs in tandem, spanning about 80 amino acids that are involved in hormone response element (HRE) recognition.[14] The LBD is usually formed by 12 α-helices numbered from helix 1 (H1) to helix 12 (H12). This domain is involved in receptor dimerisation, ligand recognition and cofactor interactions.[15] Moreover, the LBD contains the ligand-binding pocket (LBP), which binds ecdysteroids, as well as certain non-steroidal ecdysone receptor agonists, such as the DAH-based insecticides.[16] The structures of the nuclear receptors have provided important information about ligand recognition and the activation mechanism of nuclear receptors. In fact, homology models based on a comparison of the EcR-LBD with known crystal structures have been employed to determine the three-dimensional (3D) structure of the EcR-LBD,[17] and docking studies have been carried out to simulate how a candidate ligand binds to a receptor.[12,18–20]

As the molecular mode of action of MACs has not been explored in beneficial insects such as *O. laevigatus*, in this study, firstly, the side effects of methoxyfenozide, tebufenozide and RH-5849 on adults of the beneficial predatory bug *O. laevigatus* were investigated in the laboratory. Exposure of insects to a treated inert surface (worst-case laboratory test) and ingestion tests (the primarily mode of action of MACs) were performed in the present study. In a second part, the LBD of the EcR of the predatory bug was cloned and sequenced. Then, a 3D model of the OlEcR-LBD was constructed to evaluate whether it exhibits the typical canonical structure with 12 α-helices. Subsequently, a docking experiment was carried out to elucidate the interactions between the receptor ligand cavity and the natural 20E moulting hormone, and finally these were compared with the DAH-based ecdysone agonist insecticides to provide a better understanding of their selective activity on the predatory bug *O. laevigatus*.

2 MATERIALS AND METHODS

2.1 Insects

Orius laevigatus was purchased from Koppert Biological Systems SL (Thripor®; Águilas, Murcia, Spain) and fed prior to the trials with eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), also purchased from Koppert Biological Systems SL (Entofood®). *Phaseolus vulgaris* L. were used as a source of water and oviposition substrate. Groups of 30 insects were maintained in cages (12 cm diameter, 5 cm high) in a climatic chamber under the standard rearing conditions of 25 $\pm$ 2 °C, 75 $\pm$ 10% RH and a 16 : 8 (L : D) photoperiod, until the assays were done.

2.2 Chemicals

The commercially available methoxyfenozide (Runner®, 24 SC; Bayer, Madrid, Spain), tebufenozide (Mimic 2F®, 24 SC; Dow Agrosciences Ibérica SA, Madrid, Spain), as well as the technical-grade compound RH-5849 (>95% pure; Rohm and Haas, Spring House, PA) were used in the tests. A broad-spectrum insecticide, deltamethrin (Decis®, 2.5 EC; Bayer, Madrid, Spain), was used as a positive commercial standard, and distilled water was used as a control. Solutions of every product were freshly prepared prior to the assays in distilled water, based on the maximum field recommended concentrations (MFRCs) in mg active ingredient (AI) per litre.

2.3 Insect bioassays

Residual exposure: adults were exposed to pesticide fresh residues using the dismountable test cages designed by Jacas and Viñuela.[21] The floor and ceiling of the cages, consisting of glass plates (12 $\times$ 12 $\times$ 0.5 cm), were treated with the chemicals under a Potter precision spray tower (Burkard Manufacturing Co., Rickmansworth, UK), producing standard deposits of 1.75 $\pm$ 0.10 mg cm^{-2} (1.25 mL; 55 kPa), which are within the interval recommended by the IOBC validity criteria for running ecotoxicological tests on beneficial arthropods (1.5–2 mg cm^{-2}).[22] Five replicates per treatment and the control with 15 adults each were used. Adult mortality was recorded after 72 h.

In order to assess the reproductive parameters, survivors were individually sexed under a binocular microscope, and groups of three couples were transferred to ventilated plastic cages (5 cm diameter, 12 cm high). Insects were fed *ad libitum* with *E. kuehniella* eggs and provided with green beans as oviposition substrate, which were changed every 2 days for a 6 day period in total. The cumulative number of eggs per female at the end of the 6 day period was used to evaluate fecundity. The percentage of egg hatch, approximately 7 days after oviposition, was used to evaluate fertility. Four replicates were done per compound and control.

Exposure via ingestion: thin layers of *E. kuehniella* eggs placed in petri dishes (4 $\times$ 4 cm) were treated under the Potter precision spray tower, as described above. Groups of 15 adults were kept in ventilated plastic cages (12 cm diameter, 5 cm high) and fed with the treated *E. kuehniella* eggs during three consecutive days. Control groups were supplied with untreated eggs only. Effects on mortality and reproductive parameters were assessed as described above.

Mortality and reproductive parameter data [mean values and standard errors (SEM)] from each type of bioassay were analysed by ANOVA, followed by a *post hoc* least significant difference (LSD) test at the 95% confidence level using Statgraphics® Plus, v.5.0.[23]

2.4 EcR-LBD from the *Orius laevigatus* (OlEcR-LBD) sequence and phylogeny

Adults of *O. laevigatus* were used to extract total RNA using TRI reagent (Sigma-Aldrich, Bornem, Belgium), based on the single-step, liquid-phase separation method.[24] The quality and quantity of the extracted RNA were examined by gel electrophoresis and spectrophotometry using a Nanodrop™ND-1000 (Thermo Fisher Scientific BVBA, Asse, Belgium). Subsequently, first-strand cDNA was synthesised using SuperScript™II reverse transcriptase (Invitrogen NV, Merelbeke, Belgium) with the oligo$(dT)_{12-18}$ primers according to the manufacturer's protocol.

The OlEcR-LBD coding sequence was then determined through a number of PCR reaction steps. Partial sequences of the LBD were obtained using degenerate and specific primers (Table 1) located in the coding sequence of the LBD and DBD of the receptor and designed using Primer3 software.[25] Design of degenerate primers was based on known EcR sequences from different Mecoptera, Trichoptera, Strepsiptera, Coleoptera, Hymenoptera, Lepidoptera and Diptera insect species. Gene-specific primers were designed in the partial sequence obtained with the degenerate primers. PCR products were purified using the Cycle Pure kit (Omega Bio-Tek, Norcross, GA) and were then sent for sequencing (Agowa,

Table 1. Degenerate and specific primers used to complete OlEcR-LBD coding sequence

Fragments	Forward	Reverse	Product size (bp)
Fragment 1 (LBD)	5′-GAAGTNATGATGYTNMGNATG-3′	5′-ACGTCCCAKATYTCWKCNARVAA-3′	199
Fragment 2 (DBD and LBD)	5′-TGCGGHGAYMGDGCNTCYGG-3′	5′-CCGACAGAATATGAGAAGGT-3′	723
Fragment 3 (LBD)	5′-CGTAGTCGAAAACCTTCTCA-3′	5′-ACGTCCCAKATYTCWKCNARVAA-3′	280
Fragment 4 (LBD)	5′-CCATCGGCTTGTCTACTTT-3′	5′-CTTGGATCTTCTCGACTTTC-3′	417
Cloning	5′-AAGAGTCACAAAAACCAACG-3′	5′-AGCTTGAGTGAGAAGCACAT-3′	728

Berlin, Germany). Afterwards, the whole fragment was cloned and sequenced for confirmation.

The same *O. laevigatus* cDNA as used for identification of EcR was used for the initial PCR reactions of the cloning. Subsequently, the PCR products were ligated into a pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. Afterwards, plasmids were transformed in competent *Escherichia coli* XL-1 Blue Cells by heat stock, and then plated out on an ampicilin-containing lisogeny broth agar plate. After 16 h incubation, formed colonies were checked by colony PCR, and several of these positive colonies were then purified using Plasmid Mini Prep kit (Omega Bio-Tek) and sent for sequencing (Agowa).

The EcR-LBD sequences of several arthropods and two human orthologues of EcR were retrieved by Blast searches against the GenBank database. The chosen sequences were then aligned by CLUSTALW2/CLUSTALX2,[26] and the phylogenetic trees were designed using MEGA4 software.[27] Bootstrap analysis with 1000 replicates for each branch position was used to assess support for nodes in the tree.[28]

2.5 Expression of EcR in the *Orius laevigatus* nymphal stage

The presence of the EcR in *O. laevigatus* nymphs was confirmed by RT-PCR using specific primers. A mixture of different stages of *O. laevigatus* nymphs was used to extract total RNA, and subsequently cDNA was synthesised following the same procedure as described before.

2.6 Modelling of OlEcR-LBD and docking studies

Homology modelling of the OlEcR-LBD was performed with the YASARA structure program[29] running on a 2.53 GHz Intel core duo Macintosh computer. Different models were built from the X-ray coordinates of the EcR of *Heliothis virescens* F. (Lepidoptera: Noctuidae) in complex with synthetic DAH-based ligand (PDB code 3IXP), the RXR-USP receptor of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) bound to ponasterone A (P1A) (PDB Code 2NXX),[30] the EcR-LBD of the Hemiptera *B. tabaci* complexed to P1A (PDB code 1Z5X)[31] and the EcR-USP of *H. virescens* in complex with 20E (PDB code 2R40),[32] used as templates, respectively. The identity in amino acids in the LDB between OlEcR-LBD and the corresponding region of *H. virescens, T. castaneum* and *B. tabaci* is 63, 84 and 80% respectively. Finally, a hybrid model was built up from the four previous models. PROCHECK[33] was used to assess the geometric quality of the 3D model. In this respect, all of the residues of OlEcR-LBD were correctly assigned on the allowed regions of the Ramachandran plot (result not shown). Using ANOLEA[34] to evaluate the models, a single stretch of four residues (3LPVN6) over 235, corresponding to the disordered N-terminal end of OlEcR-LBD, exhibited energy over the threshold value. Molecular cartoons were drawn with YASARA and PyMol (DeLano WL, http://pymol.sourceforge.net). Docking of 20E, P1A, tebufenozide, methoxyfenozide and RH-5849 to OlEcR-LBD was performed with the YASARA structure program. Clipping planes of OlEcR-LBD complexed to 20E, P1A, tebufenozide, methoxyfenozide and RH-5849 were rendered with PyMol.

3 RESULTS

3.1 Insect bioassays

Details of the biological activity of the treatments against adults of *O. laevigatus* are given in Table 2. Residual and ingestion exposure to methoxyfenozide, tebufenozide and RH-5849 during 72 h did not cause any loss of survival ($P > 0.05$) over the controls. When exposed to deltamethrin, however, 100% of adults died after 24 h in both experiments.

In addition, no sublethal side effects of the three DAH-based insecticides on reproductive parameters were observed in either assay ($P > 0.05$). Based on these results, it was decided to investigate the mechanism of DAH selectivity towards *O. laevigatus* at the molecular level using modelling and docking experiments.

3.2 OlEcR-LBD sequence, phylogeny and expression in *Orius laevigatus*

The cDNA encoding the LBD from OlEcR-LBD was cloned in order to obtain the sequence. However, it should be noted that the N-terminal end of the LBD was not completely obtained, in spite of intensive efforts using the 3′RACE system kit (Invitrogen). The last two residues of the LBD were lacking. As almost all known EcR sequences in insect species show the exact same two amino acids in that position (DV), this consensus sequence was used to complete the sequence for the present modelling analysis. Figure 1 shows a multiple alignment with the amino acid sequence of OlEcR-LBD, together with the EcR-LBD from most other known hemipteran species, and several members from other insect orders. OlEcR-LBD exhibits some amino acid substitutions in positions where conservation is usually very high throughout the class of Insecta. These residues are marked on the figure with blue dots (residues at position 54 in helix 3, 132 in helix 7 and 227 in helix 11). In addition, amino acids involved in ligand binding are marked by red dots.[12]

As shown in Fig. 2, phylogenetic trees of the EcR-LBD, including various species from several insect orders such as Diptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera and Coleoptera, together with a number of Crustacea and Chelicerata, group OlEcR-LBD, together with the Hemiptera, close to *Nezara viridula* L. (Hemiptera: Pentatomidae) EcR-LBD. Maximum parsimony trees also confirmed this result (data not shown). Moreover, the EcR-LBDs of Hemiptera, Hymenoptera, Coleoptera, Phthiraptera, Blatodea and Orthoptera clustered away from the Mecopterida superorder (which includes Lepidoptera and Diptera orders).

Table 2. Biological activity of methoxyfenozide, tebufenozide and RH-5849 in adults of *Orius laevigatus*

Compounds	Concentration (mg AI L^{-1})	% Mortality 72 h	Fecundity (eggs $female^{-1}$ day^{-1})	% Egg hatch
Residual[a]				
Control	–	8 ± 4	7 ± 1	83 ± 3
Methoxyfenozide	96	16 ± 5	6 ± 1	78 ± 2
Tebufenozide	180	27 ± 5	5 ± 1	80 ± 2
RH-5849	180	24 ± 11	7 ± 1	81 ± 6
F (df)[b]	–	1.34 (3; 15)	0.88 (3; 12)	0.26 (3; 12)
P[c]	–	0.29	0.47	0.85
Ingestion[a]				
Control	–	13 ± 4	5 ± 1	79 ± 5
Methoxyfenozide	96	12 ± 4	4 ± 1	80 ± 7
Tebufenozide	180	7 ± 4	5 ± 1	90 ± 2
RH-5849	180	9 ± 4	4 ± 1	72 ± 2
F (df)[b]	–	0.61 (3;16)	0.94 (3;12)	2.66 (3;12)
P[c]	–	0.62	0.45	0.09

[a] The insecticide deltamethrin caused 100 ± 0% mortality at 24 h after the treatment.
[b] Fisher test (degrees of freedom).
[c] Probability.

RT-PCR from RNA extracted from mixed nymphal stages of *O. laevigatus* also confirmed the expression of the EcR gene in this developmental stage.

3.3 Modelling of OlEcR-LBD and docking studies

The EcR-LBD of the predatory bug *O. laevigatus*, as modelled from the X-ray coordinates of different insect LBDs, exhibited the canonical 3D conformation of the LBD of arthropod EcR built up of twelve α-helices (labelled H1 – H12) associated with a short hairpin of two β-strands β1 and β2 (Fig. 3A). The model strikingly resembles that of *T. castaneum* RXR-USP used as one of the templates (PDB code 2NXX) (Fig. 3B), even though the loop that connects α-helix H2 to α-helix H3 was not correctly X-ray solved and is lacking from the 3D structure of the RXR-USP. Helices H2, H3, H5, H8 and H11 in OlEcR-LBD delineated a ligand-binding cavity that usually accommodates the natural insect moulting hormone 20E (Fig. 3C) and also the P1A molecule (Fig. 3E). Docking experiments performed with these two ecdysteroids yielded a typical H-bonding scheme that the OlEcR-LBD shares with other arthropod EcR-LBDs. Both 20E and P1A interacted with the LBD pocket via a network of five hydrogen bonds, involving the hydrophilic residues glutamic acid 14, threonine 44, threonine 47, alanine 102 and tyrosine 112 (Figs 3D and F). In addition, hydrophobic and stacking interactions occur with hydrophobic (methionine 209, cysteine 210, methionine 107) and aromatic residues (phenylalanine 101, tyrosine 107, tryptophan 228) located at the periphery of the ligand-binding cavity to complete the ligand anchorage into the pocket.

However, upon docking of tebufenozide (Fig. 3G) to the ligand-binding cavity of the OlEcR-LBD, more or less severe steric hindrances were shown to occur, especially with residues Met85, Val120, Leu125, Gln205, Asn206 and Met209, which form the wall of one of the two lobes of the ligand-binding cavity in which the ethyl-phenyl ring of tebufenozide becomes inserted. Also, for the closely related methoxyfenozide, a rather similar steric hindrance was observed upon docking of the ecdysone agonist in which the methoxy group of the agonist becomes inserted (Fig. 3H). Upon docking of the agonist RH-5849 (Fig. 3I), a less severe steric hindrance occurs with the ligand-binding cavity, involving residues Met85, Leu124 and Asn206, owing to the lack of any substituent linked to the phenyl ring of this agonist.

These docking experiments suggest that DAH-based insecticides such as tebufenozide, methoxyfenozide and RH-5849 are likely to exert no deleterious effect on the predatory bug *O. laevigatus*, which supports previous reported experimental data from biological activity tests performed with both insecticidal molecules.

4 DISCUSSION

The DAH-based ecdysone agonist insecticides are active against several economically important lepidopteran and coleopteran pests and are considered to be environmentally friendly compounds with an excellent margin of safety to non-target organisms.[6,7] Consequently, these pesticides are usually included in IPM programmes targeting different pests and crops all over the world.

Based on their mode of action, a significant effect on survival must not be expected when adults of natural enemies are exposed to pesticide residues or fed with treated diet. In the literature there are many studies supporting the compatibility of both adults and nymphs of predatory Hemipterans with MAC pesticides.[35–40] In agreement with this, in the present study, methoxyfenozide, tebufenozide and RH-5849 were harmless to adults of *O. laevigatus* in the short term. Other beneficials belonging to different insect orders, as well as pollinators, seem also to be compatible with MAC pesticides.[41–45]

The MACs, besides their role in metamorphosis, are also essential in insect reproduction by taking part in vitellogenesis, ovulation and spermatocyte growth.[46] In the present study, the reproduction capacity of *O. laevigatus* females was similar to that of the control, which is in agreement with previous studies on this species.[39,47–49] However, reproductive disturbances, such as a lower fecundity in *Deraecoris brevis* (Uhler) (Hemiptera: Miridae)[50] or a higher number of egg batches in *Picromerus bidens* L. (Hemiptera: Pentatomidae), were observed after methoxyfenozide exposure.[51]

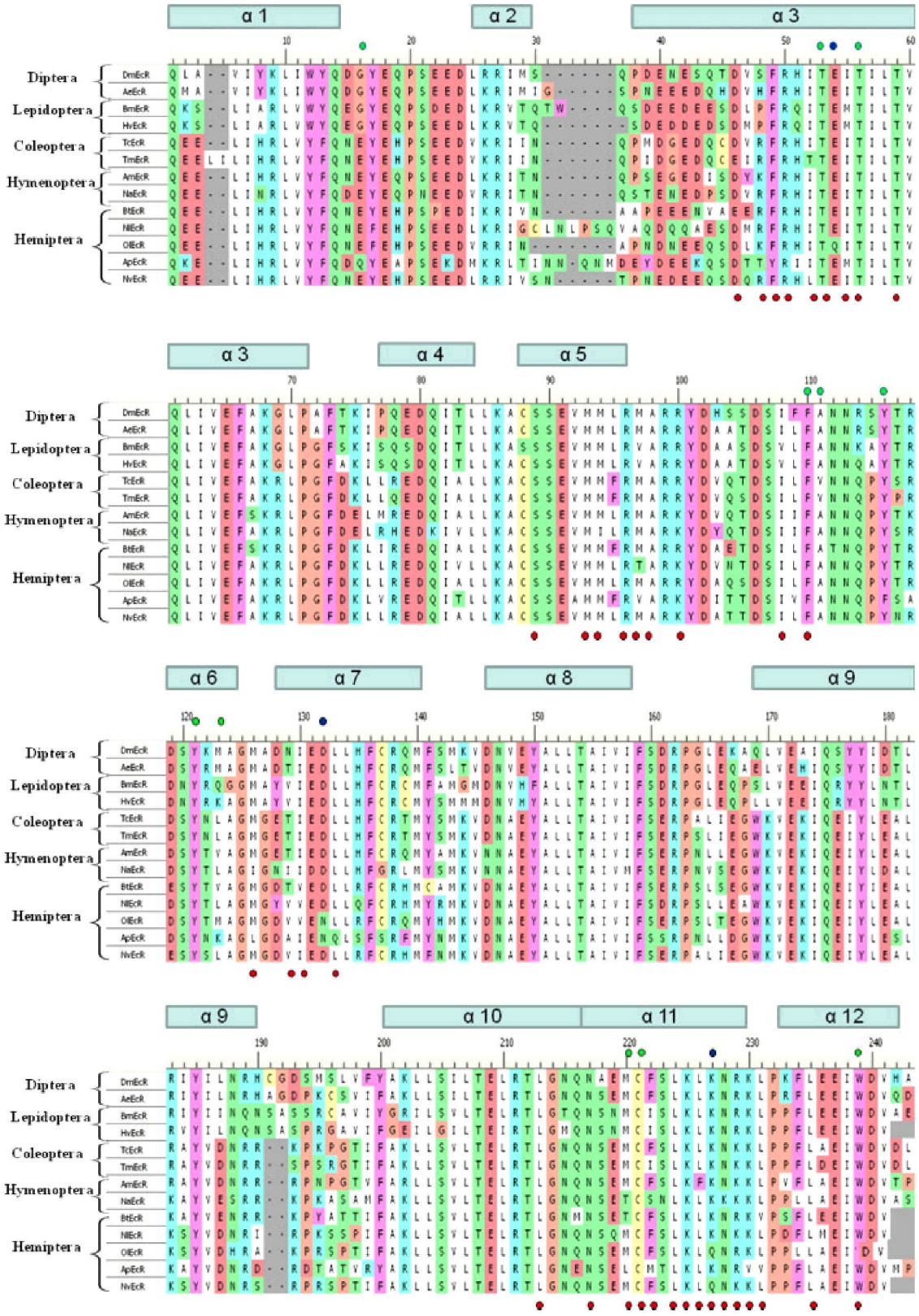

Figure 1. Sequence alignment of ecdysone receptor ligand-binding domains (EcR-LBD), including OlEcR-LBD. The alignment includes EcR from Diptera, Lepidoptera, Coleoptera, Hymenoptera and Hemiptera. The organism abbreviations are: Diptera (DmEcR: *Drosophila melanogaster*; AaEcR: *Aedes aegypti*); Lepidoptera (BmEcR: *Bombyx mori*; HvEcR: *Heliothis virescens*); Coleoptera (TcEcR: *Tribolium castaneum*; TmEcR: *Tenebrio molitor*); Hymenoptera (AmEcR: *Apis mellifera*; NaEcR: *Nasonia vitripennis*); Hemiptera (BtEcR: *Bemisia tabaci*; NlEcR: *Nilaparvata lugens*; OlEcR: *Orius laevigatus*; ApEcR: *Acyrthosiphon pisum*; NvEcR: *Nezara viridula*). Blue dots indicate amino acid substitutions in the OlEcR-LBD sequence in positions where conservation is usually very high throughout the insect class. Red dots indicate the amino acids involved in ligand binding in the EcR-LBD.[12] Green dots indicate hydrophilic, hydrophobic or aromatic residues involved in the ligand anchorage into the pocket. The figure has been prepared using CINEMA.

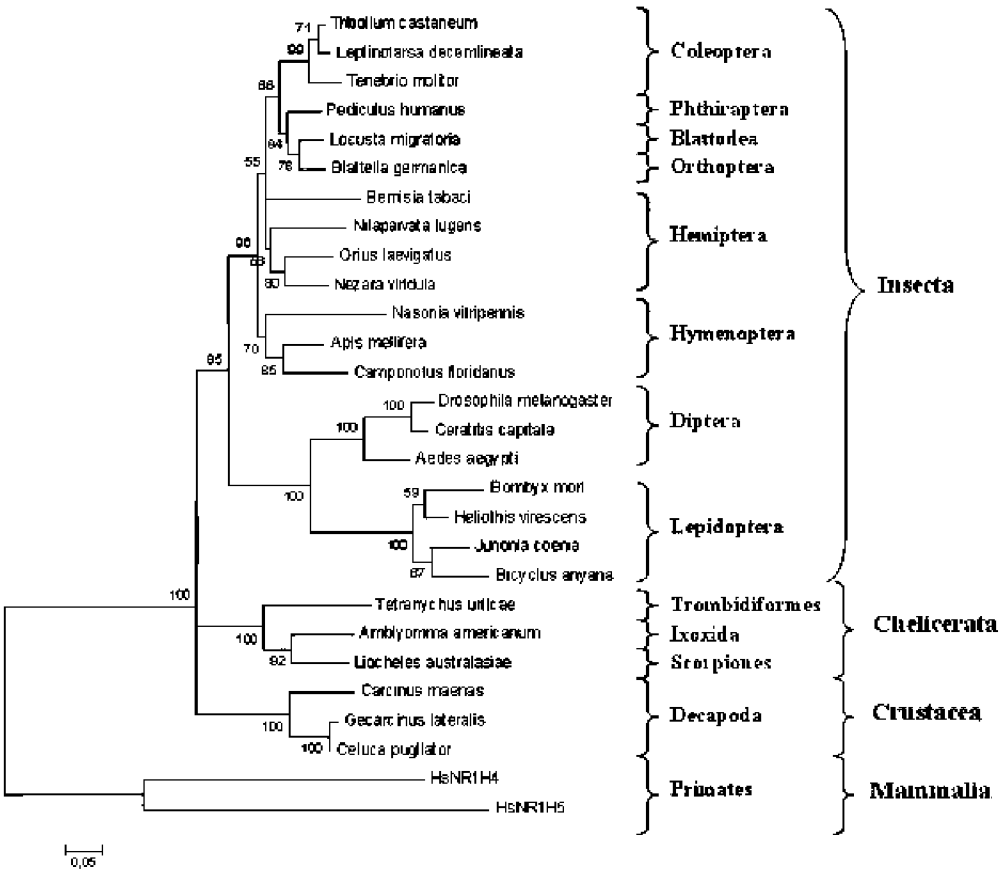

Figure 2. Phylogenetic tree of EcRs. This tree was constructed by the neighbour-joining method using the amino acid sequences of the LBD of the selected sequences. Bootstrap values as a percentage of 1000 replicates of >50 are indicated on the tree.

The pharmacokinetics and metabolic detoxification of compounds could play an important role in determining the biological spectrum of DAH-based ecdysone agonists.[43,52,53] The latter molecular studies are consistent with the concept that an important process in the selectivity of these compounds is the specific binding of the MACs to the target EcR, which is governed by a lock-and-key principle.[7] For instance, the binding affinity is high in targeted Lepidoptera pests, whereas binding is low or not detectable in non-targeted insects.[54] In agreement with these results, previous studies have found tebufenozide to have an extremely low affinity for hemipteran EcRs belonging to the Sternorrhyncha, namely *B. tabaci* and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae).[10,31] In this respect, it appears that differences in the architecture of LBP may provide the differential binding affinities of the DAH-based compounds among different taxonomic orders.[31] Consequently, for those non-sensitive insects such as *O. laevigatus*, it is expected that differences at LBP level may hinder MAC binding.

The amino acid residues involved in ligand binding with the OlEcR-LBD are indicated in Fig. 1. OlEcR-LBD exhibits a high conservation among these ligand-binding-involved residues by comparison with other insects that show no or low susceptibility for MACs. In contrast, in Lepidoptera, which show a high sensitivity for tebufenozide and methoxyfenozide, it was observed that the residues isoleucine 55, methionine 97 and isoleucine 108 are replaced by methionine 55 and two valine residues (97, 108) respectively. In particular, the presence of an isoleucine at position 55 in non-sensitive species generates steric hindrance between the γ-methyl group of the isoleucine residue and the C5-methyl group at the B-ring of the tebufenozide or the C4-ethyl group of its B-ring, depending on the orientation of the tebufenozide molecule.[17] In addition to these differences, other substitutions were observed in amino acids involved in the ligand binding. Proline 48, isoleucine 222 and lysine 227 are conserved in lepidopteran BmEcR and HvEcR, but replaced in OlEcR by amino acids that are structurally different: lysine 48, phenylalanine 222 and glutamine 227 (Fig. 1). Moreover, the present docking results suggest that, owing to the restricted extent of the ligand-binding cavity, a steric clash occurred upon docking of tebufenozide, methoxyfenozide and RH-5849 to the ecdysone-binding site of the LBD (Figs 3G, H and I). An examination of the amino acid composition surrounding the restricted cavity made it possible to identify those amino acids that may play a role in this restriction. In particular, residues threonine 129 and isoleucine 130, present in TcEcR and TmEcR, and isoleucine 130, present in BmEcR and HvEcR, are replaced by valine in *O. laevigatus* (Fig. 1). It is suggested that these combined differences could explain the lack of toxicity of the DAHs against the predatory bug *O. laevigatus*.

However, it should also be noted here that, owing to the intrinsic flexibility of the amino acids involved in the steric hindrance with tebufenozide, methoxyfenozide and RH-5849, some accommodation of the DAH agonists to the ligand-binding cavity of OlEcR-LBD should be achieved, which could prevent the most severe steric clash from occurring. In this respect, the binding of ecdysone to the ultraspiracle protein from *Heliothis virescens*[55] was shown to depend on an induced fit mechanism. The plasticity

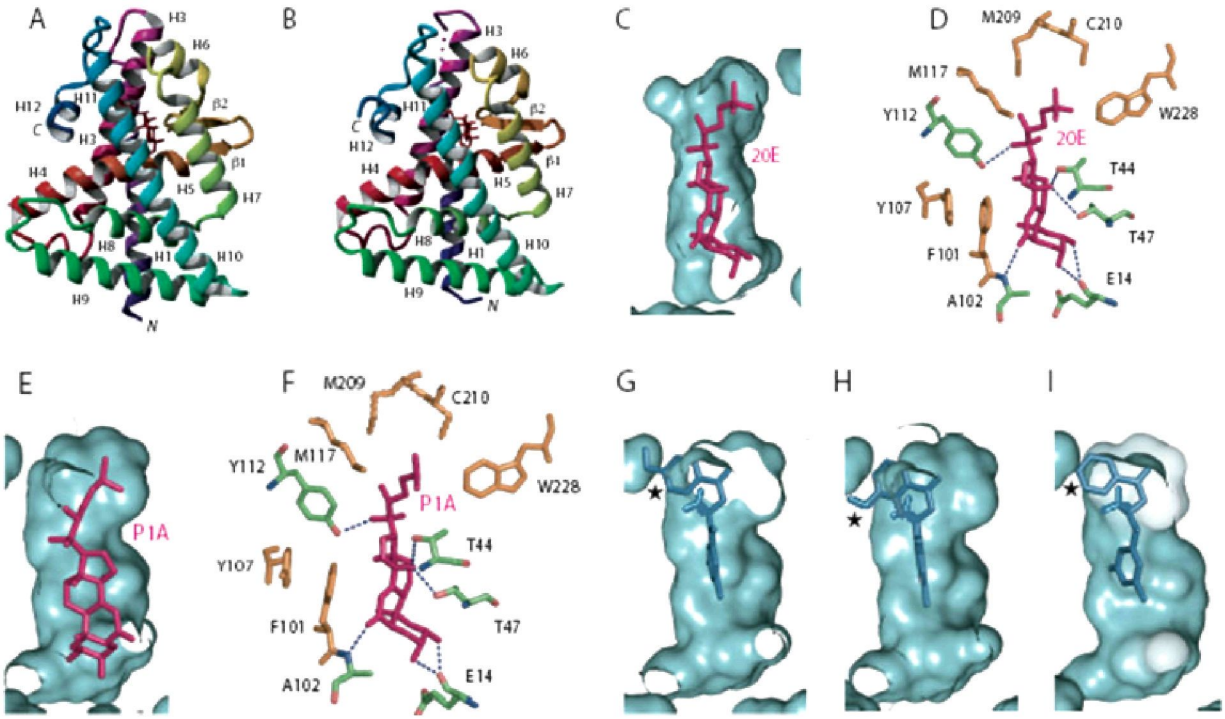

Figure 3. Overall 3D conformation of the modelled EcR-LBD domain of the predatory bug *Orius laevigatus* (A) compared with the LBD structure of the RXR-USP of *Tribolium castaneum* (B). The twelve α-helices distributed along the polypeptide chain are numbered H1–H12, and the two β-strands β1 and β2 forming a protruding hairpin motif are indicated. N and C indicate the N-terminal and C-terminal ends of the polypeptide chain respectively. (C) Clip view across the ligand-binding pocket of the OlEcR-LBD harbouring 20-hydroxyecdysone (20E) (pink stick). (D) Network of hydrogen bonds (dashed dark lines) anchoring 20E to the OlEcR-LBD. Hydrophobic and aromatic residues interacting with the ligand by hydrophobic and stacking interactions, respectively, are coloured orange. Residues are labelled according to the 3D model built for the OlEcR-LBD. (E) Clip view across the ligand-binding pocket of the OlEcR-LBD harbouring ponasterone A (PA1) (pink stick). (F) Network of hydrogen bonds (dashed dark lines) anchoring P1A to the LBD. Hydrophobic and aromatic residues interacting with the ligand by hydrophobic and stacking interactions, respectively, are coloured orange. Residues are labelled according to the 3D model built for OlEcR-LBD. (G) Clip view across the ligand-binding pocket of the OlEcR-LBD harbouring tebufenozide (blue stick). Note the steric clash ( ) of the ethyl group at the phenyl ring of tebufenozide with the wall of the ligand-binding pocket. (H) Clip view across the ligand-binding pocket of the OlEcR-LBD harbouring methoxyfenozide (blue stick). Note the steric clash ( ) of the methoxy group at the phenyl ring of methoxyfenozide with the wall of the ligand-binding pocket. (I). Clip view across the ligand-binding pocket of the OlEcR-LBD harbouring RH-5849 (blue stick). Note the steric clash ( ) of the phenyl ring with the wall of the ligand-binding pocket, which is less severe compared with tebufenozide and methoxyfenozide.

responsible for the promiscuous character of the ligand-binding pocket for different agonists and antagonists was further stressed by Billas *et al.*[56–58] As a result, the steric discrepancies occurring upon binding of tebufenozide, methoxyfenozide and RH-5849 to the binding pocket of OlEcR-LBD are an important but probably not the only explanation for the lack of toxicity of these agonists to *O. laevigatus*.

In conclusion, this paper reports on the effects of DAH-based ecdysone agonists on *O. laevigatus*. The data show no biological activity of these compounds on the predatory bug. Furthermore, modelling of the OlEcR-LBD and docking experiments also suggest the absence of binding and activating EcR, and, consequently, the absence of toxic effects caused by disruption of EcR signalling on this important predatory natural enemy. Thus, methoxyfenozide, tebufenozide and RH-5849 could be applied safely in IPM programmes in which *O. laevigatus* was present, although further studies are recommended to test DAHs on other auxiliary fauna to prevent undesirable effects.

ACKNOWLEDGEMENTS

This study was supported by the Spanish Ministry of Science and Innovation (SMSI) (projects AGL2010-22196-C02-01 and -02 to Drs A and E Viñuela) and by the Special Research Fund of Ghent University and the Fund for Scientific Research – Flanders (FWO-Vlaanderen, Belgium) to Dr G Smagghe. F Amor is the recipient of a PhD grant from the Spanish Ministry of Science and Innovation.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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